Comparison of Proliferation and Differentiation Capacity of Human Adipocyte Precursor Cells From the Omental and Subcutaneous Adipose Tissue Depot of Obese Subjects

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Upper body obesity is characterized by an expansion of the visceral adipose tissue and is associated with an increased susceptibility for type 2 diabetes and cardiovascular disease. In order to get a better understanding of the regulation of body fat distribution, the aim of the present study was to compare adipocyte development between the omental and subcutaneous adipose tissue region in obese subjects. Therefore, the proliferation and differentiation capacity in primary cultures of adipose tissue-derived stromal cells were compared between the 2 depots in a group of 29 obese individuals, of which 21 were women. Proliferation of the cells was stimulated using fetal calf serum (FCS) and assessed by counting the cell number in the culture dishes. Differentiation of preadipocytes was assessed in parallel by morphological criteria and determination of glycerol-3-phosphate dehydrogenase (GPDH) after stimulation by standardized adipogenic conditions. Stromal cells from the subcutaneous adipose tissue region proliferated faster (doubling time, 4 ± 1 days) than those from the omental region (doubling time, 5 ± 1 days), whereas there was no regional difference in adipose differentiation with any of the adipogenic media. The same findings were observed when men were excluded from the analysis. Interestingly, there were more endothelial cells in the cultures from the omental tissue as compared to those from the subcutaneous tissue, but there was no correlation between endothelial cell contamination and proliferation capacity, suggesting that the regional difference in proliferation capacity was not due to regional differences in the amount of endothelial cells. In addition, we found a negative correlation between donor age and proliferation of subcutaneous cells but not of omental cells, possibly explaining the greater capacity for adipose tissue growth in the omental as compared to the subcutaneous depot with aging. In conclusion, there may exist regional differences in adipose tissue growth with regard to proliferation capacity, whereas there are apparently no significant differences in in vitro differentiation capacity between subcutaneous and omental preadipocytes.

MANY EPIDEMIOLOGICAL and experimental studies of the last decades have revealed an essential role of the anatomical distribution of adipose tissue for the development of metabolic abnormalities, in particular in obese subjects. Upper body obesity or an abdominal type of body fat distribution correlates with an increased susceptibility for metabolic disturbances such as glucose intolerance, hyperinsulinemia, dyslipidemia, and hypertension.[1,2] This link appears to be principally due to heterogeneity of adipose tissue from different regions, in particular due to differences in the metabolic function between the adipose tissue depots. For example, compared with subcutaneous adipocytes, visceral or omental adipocytes are relatively resistant to the antilipolytic action of insulin but more sensitive to the stimulation of lipolysis by catecholamines.[3] Therefore, an expanded omental adipose tissue may be associated with elevated free fatty acid levels in the circulation, which in turn contributes to insulin resistance in liver and skeletal muscle by several mechanisms, as well as impairs insulin secretion from the pancreas.[2,3] In addition, it is possible that differences in the endocrine function between the various adipose regions play an important role in the metabolic abnormalities in subjects with upper-body obesity.[4]

Although many studies have been performed on regional differences in adipose tissue metabolism or endocrine function, only very limited information is available on regional differences in adipocyte development.[5-9] However, this kind of information is important for a better understanding of how body fat distribution is regulated and to develop new strategies to interfere with adipose tissue growth, particularly in the dangerous intra-abdominal depots. The aim of the present study was to examine the influence of the anatomical site on the proliferation of human preadipocytes and the formation of new adipocytes. For this purpose, we compared the proliferation capacity and in parallel the differentiation capacity between stromal cells obtained from the omental and subcutaneous adipose tissue of obese subjects.

MATERIALS AND METHODS

Subjects

Human adipose tissue was obtained from the abdominal subcutaneous region (epigastric region of the abdominal wall) and omental region (omentum major) from 29 obese (body mass index [BMI], 46.1 ± 7.2 kg/m^2, mean ± SD), but otherwise healthy subjects (8 men, 21 women) undergoing weight-reducing surgery (adjustable gastric banding). Except for obesity and minor metabolic disturbances, the subjects were healthy and took no regular medication. Informed consent was obtained from the subjects before the surgical procedure. The study protocol has been approved by the Ethical Committee of the University of Düsseldorf. After being removed, adipose tissue samples of 5 to 20 g were immediately transported to the laboratory.

Preadipocyte Isolation From Abdominal Subcutaneous and Omental Adipose Tissue

The omental and subcutaneous adipose tissue specimens were separately dissected from fibrous material and visible blood vessels, weighed, minced into small pieces, and digested in phosphate-buffered saline (PBS) containing 20 mg/mL bovine serum albumin (BSA) and 250 U/mL collagenase (Biochrom AG, Berlin, Germany) for 90 min-

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Table 1. Characteristics of the Subjects

	No. of subjects	Mean ± SD	Range
Gender	8 men, 21 women	—	—
BMI (kg/m^2)	29	46.1 ± 7.2	31.8-68.4
Age (yr)	29	36 ± 10	17-61

utes at 37°C. The completely disaggregated tissue was centrifuged for 10 minutes at 200 × *g* and the pellet of stromal cells (ie, preadipocytes) was resuspended in an erythrocyte lysis buffer (consisting of 0.154 mol/L NH_4Cl, 5.7 mmol/L K_2HPO_4, and 0.1 mmol/L EDTA, pH 7.3) to remove contaminating red blood cells, filtered through a polypropylene mesh (pore size 150 μm) and centrifuged for 10 minutes at 200 × *g*. The cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, filtered again (pore size 70 μm), and the total cell number determined microscopically using a Neubauer chamber. Using the total number of cells and the weight of the adipose tissue specimens, the number of stromal cells per gram of adipose tissue was calculated for both adipose tissue regions. Finally, the cells were seeded in 12-well plates for either differentiation or proliferation experiments.

Assessment of Preadipocyte Proliferation

For 24 subjects, cells were seeded in a density of 10,000 cells/cm^2 and incubated in DMEM/F-12 medium, containing 50 μg/mL gentamycin and 10% fetal calf serum (FCS). Cell proliferation was assessed by counting the cell number in the growing cultures at 24-hour intervals until day 7. After day 7, most of the cultures were confluent and contact inhibition occurred. The cell number of 5 randomly selected areas (each 1 mm^2) in 4 wells was counted under the microscope (ie, 20 counts on a single sample). Proliferation was expressed as percentage of the number of cells counted 24 hours after inoculation. Three persons were involved in the counting of the cell number; a single sample was counted by 1 person. The coefficient of variation on a single sample was calculated to be 30%. In 5 cases, a single sample was counted by the 3 persons separately; from this the intraindividual coefficient of variation was calculated and this appeared to be 3%.

Assessment of Preadipocyte Differentiation

For all subjects, cells were seeded in a density of 33,000 cells/cm^2 and incubated in DMEM/F-12 medium, containing 50 μg/mL gentamycin and 10% FCS (Biochrom AG, Berlin, Germany) for 20 hours at 37°C in 5% CO_2 to become attached. Then, cells were washed twice with PBS and differentiation was started, using 3 different chemically defined, serum-free media, in 3 wells for each medium.[10] The first medium consisted of DMEM/F-12 (50:50, vol:vol) with 10 μg/mL transferrin, 66 nmol/L insulin, 200 pmol/L triiodothyonine (T_3), and 50 μg/mL gentamycin. The second medium contained, in addition to the ingredients of the first medium, cortisol at a concentration of 100 nmol/L. The third medium contained, in addition to the ingredients of the second medium, 3-isobutyl-1-methylxanthine (IBMX) at a concentration of 500 μmol/L. IBMX was added to the medium only the first 3 days. The cells were cultured for 16 days and the medium was renewed every 2 to 3 days.

On day 16, differentiation was assessed by quantifying glycerol-3-phosphate dehydrogenase (GPDH) activity using an established procedure.[11] Cells were washed with PBS and harvested in prechilled 50 mmol/L Tris-HCl buffer containing 1 mmol/L EDTA and 1 mmol/L mercaptoethanol. After sonification, aliquots of the cell extracts were added to an assay mixture containing 100 mmol/L triethanolamine HCl buffer (pH 7.5), 2.5 mmol/L EDTA, 0.12 mmol/L NADH, and 0.1 mmol/L mercaptoethanol, and GPDH activity was measured spectrophotometrically at 340 nm. The reactions were started by adding 0.2 mmol/L dihydroxyacetonphosphate. GPDH was expressed as milliunits per milligram total protein. The protein concentration in the cell extracts was measured according to a modification of the method described by Lowry et al,[12] using a precipitation step with 6% (vol/vol) trichloroacetic acid to avoid interference with lipids.[13] BSA was used as a protein standard.

The percentage of differentiated cells was determined for each differentiation condition by counting differentiated and undifferentiated cells in 5 randomly selected areas (millimeters squared) under the microscope. Differentiated preadipocytes were distuiguished from the other cells because of their characteristic multiple cytoplasmatic lipid droplets. Furthermore, the number of endothelial cells was counted in the same selected areas to determine the percentage of endothelial cells in the cultures. Endothelial cells could be easily distuiguished from the fibroblast-like preadipocytes, due to their specific morphology. In order to verify that these cells were endothelial cells, the presence of factor VIII (von Willibrand factor) antigen was shown by immunohistochemical staining, using a factor VIII antibody, which binds specifically with the cytoplasm of human endothelial cells (data not shown).

Statistics

Values are given as mean ± SD. A linear regression analysis (single) was used to analyze correlations. Statistical differences were analysed using Student's paired and unpaired *t* tests. Statistical analysis was performed using SPSS 9.0 statistical software package (SPSS Inc, Chicago, IL).

RESULTS

Some clinical characteristics of the study group are listed in Table 1. The group consisted of 29 subjects of which 21 were women. All subjects were obese with a BMI ranging from 31.8 to 68.4 kg/m^2. The age range was from 17 to 61 years.

Proliferation Capacity in Omental and Subcutaneous Preadipocytes

For 24 subjects, proliferation of the inoculated cells was stimulated by 10% FCS. Proliferation was expressed as percentage of the number of cells counted at day 1. Figure 1 shows

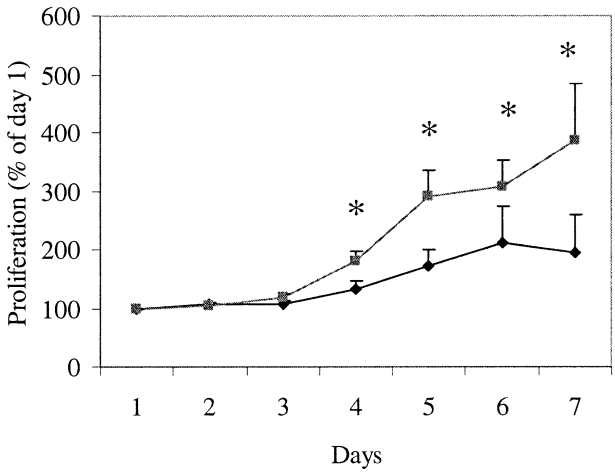

Fig 1. Comparison of proliferation rate between omental (◆) and subcutaneous (■) stromal vascular cells of obese individuals. Data represent percent increase ± SD compared to day 1. *$P < .05$ (paired *t* test). Number of subjects = 24.

Table 2. Comparison of Number of Stromal Vascular Cells per Gram Adipose Tissue, and Differentiation Capacity Between Omental and Subcutaneous Adipose Tissue From Obese Individuals

	Omental	Subcutaneous	*t* Test OM *v* SC (*P* value)
No. of stromal vascular cells per g adipose tissue	869,829 ± 382,028	657,521 ± 444,942	<.05
No. of stromal vascular cells corrected for no. of endothelial cells	774,768 ± 451,640	657,521 ± 444,942	NS
GPDH (standard)	78.2 ± 124.8	171.0 ± 329.8	NS
GPDH (cortisol)	177.7 ± 166.9	233.0 ± 389.5	NS
GPDH (cortisol + IBMX)	342.2 ± 350.6	371.0 ± 430.9	NS
% differentiated cells (cortisol)	8.2 ± 5.9	8.9 ± 11.1	NS
% differentiated cells (cortisol + IBMX)	18.2 ± 12.6	16.0 ± 13.5	NS

NOTE. Data are presented as mean ± SD. Differentiation capacity was expressed as specific GPDH activity (mU/mg protein). Number of subjects = 29. The number of stromal vascular cells per gram of adipose tissue was determined directly after the isolation of these cells out of the adipose tissue, when the total yield of cells was determined (see Methods). GPDH and percent differentiation were determined in cells cultured for 16 days in a serum-free, chemically defined adipogenic medium (see Methods for more details).

the comparison of the proliferation rate between omental and subcutaneous cells from the same donors. The cells from both regions started to increase in number from day 4 (paired *t* test *v* day 1, $P < .05$). However, subcutaneous cells proliferated at a higher rate than omental cells. This difference was statistically significant between day 4 and 7 (paired *t* tests, $P < .05$). The doubling time, calculated from the day after inoculation, was for the subcutaneous cells 4 ± 1 days, whereas for the omental cells it was 5 ± 1 days (paired *t* test, $P < .05$). It should be noted that the curves in Fig 1 are not exponential growth curves since they show the proliferation of cells in primary culture and these cells need to recover after the isolation procedure before their proliferation starts.

Differentiation Capacity in Omental and Subcutaneous Preadipocytes

For all individuals, adipose differentiation of the cells was stimulated using either cortisol or a combination of cortisol and IBMX in a serum-free, chemically defined adipogenic medium. Table 2 shows that, for preadipocytes from both regions, the percentage of newly developed cells and GPDH activity were highest in the adipogenic medium supplemented with both cortisol and IBMX (paired *t* tests, $P < .05$). There were no differences in percentage of newly developed cells and GPDH activity between the omental and subcutaneous preadipocytes, with any differentiation media used (Table 2, GPDH or percent differentiation omental *v* subcutaneous; difference not significant [NS]). There was a positive correlation between the omental and subcutaneous GPDH activity with the 3 differentiation media used (data not shown; *r* values ranging from 0.39 to 0.58, each $P < .05$).

Effect of BMI and Age on Proliferation and Differentiation

The relationship between age or BMI on the one hand and differentiation or proliferation capacity of the omental and subcutaneous cells on the other hand was investigated. BMI did not correlate to GPDH activity or percentage of differentiation in any of the differentiation media, nor proliferation percentage from days 4 until 7 of cells from both adipose tissue regions. There was no correlation between age and GPDH activity in omental or subcutaneous cells differentiated in any of the adipogenic media. However, age correlated negatively to the percentage of proliferation from days 4 until 7 of the subcutaneous cells but not of the omental cells (subcutaneous: *r* values ranging from −0.37 to −0.65, $P < .05$). Figure 2 shows the negative correlation between age and percentage of proliferation at day 4 for the subcutaneous cells as a representative for the correlations between age and subcutaneous cell proliferation between days 4 and 7.

The effects of outliers was analyzed in a linear regression analysis. When outliers were omitted in the statistical analysis, similar results were obtained as above.

Relationship Between Cell Proliferation and Differentiation

For the omental but not for the subcutaneous cells, there were negative correlations between the percentage of proliferation from days 4 until 7 on the one hand and GPDH activity obtained using the various differentiation media on the other (*r* values ranging from −0.38 to −0.65, $P < .05$). For omental cells, the correlation between percentage of proliferation at day 4 and GPDH activity in differentiation medium containing

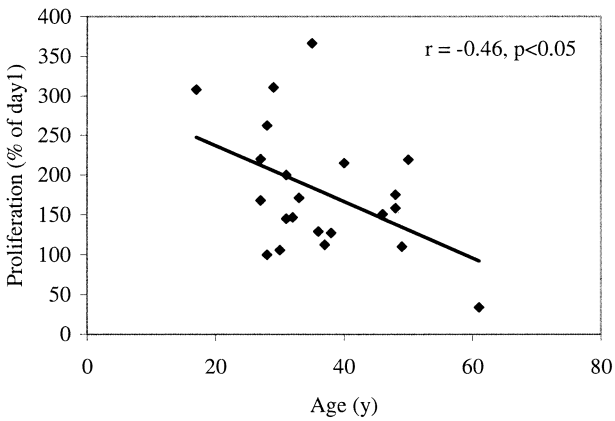

Fig 2. Relationship between age and proliferation rate of subcutaneous stromal vascular cells from 22 obese subjects. The proliferation rate was determined at day 4. Values were compared using linear regression analysis. (The proliferation rate was studied in 24 subjects. For 2 subjects it was not possible to determine this value since the cells were confluent at day 4 and therefore uncountable.)

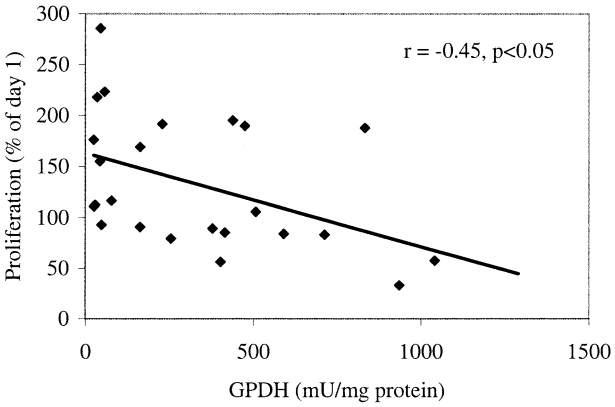

Fig 3. Relationship between proliferation rate and differentiation capacity of omental stromal cells from 24 obese subjects. The proliferation rate was determined at day 4 and GPDH activity was measured in the omental cells when differentiation was stimulated using both cortisol and IBMX. Values were compared using linear regression analysis.

cortisol and IBMX is shown in Fig 3 as one representative ($r = -0.45$, $P < .05$).

Endothelial Cell Number Assessment

Table 2 shows that the number of stromal vascular cells per gram adipose tissue was higher in the samples from the omental region than from the subcutaneous region. In order to correct the total number of stromal cells per gram of adipose tissue for any contamination by endothelial cells, the percentage of endothelial cells was assessed by counting the number of these cells in relation to the total number of cells in the cultures differentiated with medium containing either cortisol or cortisol and IBMX. The mean percentage (mean ± SD) of endothelial cells in the cultures obtained from omental adipose tissue, with both differentiation media, was 10 ± 16% (mean ± SD). Approximately 65% of the omental cultures of the subjects showed less than 5% endothelial cells. The cultures obtained from subcutaneous adipose tissue contained a negligible number of endothelial cells. The percentage of endothelial cells for every experiment was subtracted from the total number of stromal vascular cells obtained per gram of adipose tissue. Table 2 shows that the number of stromal cells per gram of adipose tissue corrected for the number of endothelial cells no longer differed between the 2 adipose tissue regions.

There were no correlations between on the one hand percentage of endothelial cells in the omental cultures and on the other hand the percentage of proliferation of the omental stromal cells or GPDH activities of the omental preadipocytes.

Effect of Gender on Proliferation and Differentiation

The subject group was split in men and women. There were no differences between men and women with regard to BMI, age, number of stromal vascular cells per gram of omental or subcutaneous adipose tissue, and proliferation capacity of the omental and subcutaneous cells (data not shown; unpaired *t* tests, NS). The GPDH activity and percentage of differentiation of the subcutaneous but not of the omental cells was significantly higher for the men than for the women (data not shown, unpaired *t* tests, $P < .05$). For the women, but not for the men, the subcutaneous cells proliferated at a higher rate as from day 4 than the omental cells (data not shown; paired *t* tests, $P < .05$), and there were negative correlations for omental cells between the percentage of proliferation on day 4 until day 7 on the one hand and GPDH activity obtained using the various differentiation media on the other hand (data not shown; *r* values ranging from -0.48 to -0.72, $P < .05$). The negative correlation between age and percentage proliferation remained in the women (data not shown; percent proliferation at day 5 *v* age, $r = -0.60$, $P < .05$). For both genders there were no differences in differentiation capacity between the omental and subcutaneous cells (data not shown; paired *t* tests, NS).

Two of the women were postmenopausal. Similar results were obtained as above when these 2 women were excluded from the statistical analysis (data not shown).

DISCUSSION

The aim of the present study was to compare both preadipocyte proliferation and differentiation capacity between the omental and subcutaneous stromal vascular cell fraction from obese individuals, in order to get a better understanding of regional adipose tissue accumulation. We found that cells from the subcutaneous adipose tissue region from obese subjects proliferated faster than those from the omental region in medium containing 10% FCS. This observation is in contrast to previous studies by Roncari et al[14] and Petterson et al,[5] respectively, in which no differences in replication rate between omental and subcutaneous adipose tissue were found. One reason for the discrepancy between the current study and the former studies may be differences in the methodological approach. For instance, in the study of Petterson et al[5] replication rates were investigated in subcultures of adipocyte precursors, whereas in the current study primary cultures were investigated. We have observed that stromal cells in subcultures proliferate at a decreasing rate as compared to cells in primary cultures (unpublished data).

We were unable to detect regional differences in preadipocyte differentiation when omental and subcutaneous cells were stimulated to undergo adipose conversion in a standard adipogenic medium or an adipogenic medium containing either cortisol or a combination of cortisol and IBMX. This finding is in disagreement with a former study from our group,[6] which showed a higher differentiation capacity in primary preadipocyte cultures of the subcutaneous abdominal region as compared to cultures from the omental region. In that study, differentiation was stimulated using cortisol and IBMX. However, in addition to differences in the BMI of the 2 study groups, the present study was performed under serum-free conditions, whereas 10% FCS was present in the former study. FCS is well known to exert a potent antiadipogenic activity.[10] A lack of regional difference in preadipocyte differentiation has also been shown in other studies on primary preadipocyte cultures of smaller groups of obese[15] and non-obese plus obese subjects.[8] In these 2 studies, preadipocyte differentiation was stimulated using a serum-free adipogenic medium that in addition to cortisol and IBMX also contained rosiglitazone or carba-

prostacyclin, respectively. In the current study, we used 3 different serum-free media for stimulation of preadipocyte differentiation, because regional discrepancies might exist in the hormonal responsiveness to various components in the media. Our findings suggest an equal sensitivity to cortisol and IBMX in the omental and subcutaneous preadipocytes with regard to differentiation, at least in obese subjects. Taken together, our current and former results suggest that the capacity to form new adipocytes is rather equal for the subcutaneous and omental adipose tissue regions. However, these studies cannot exclude that there is variation in differentiation between other regions than the omental and subcutaneous regions. A moderate regional difference in preadipocyte differentiation has been observed before between cells obtained from femoral and abdominal subcutaneous adipose tissue.[7]

The number of stromal vascular cells per gram of adipose tissue was higher in the tissues from the omental than from the subcutaneous region. We assume that this difference is due to a higher number of endothelial cells in the omental than in the subcutaneous stromal vascular fraction. The percentage of endothelial cells was microscopically assessed in the cell cultures of both regions and it was observed that the omental cultures contained on average approcximately 10% endothelial cells, whereas the subcutaneous cultures were free or contained only a negligible amount of endothelial cells. This is in agreement with a previous immunohistochemical analysis.[16] When the total number of stromal vascular cells was corrected for the number of endothelial cells, no difference in stromal cell number per gram of adipose tissue was observed between the omental and subcutaneous region.

It was analyzed whether there was a relationship between the percentage of endothelial cells in the omental cultures on the one hand and the percentage of proliferation of the stromal cells or the preadipocyte differentiation capacity on the other hand. In other studies it has been demonstrated that factors secreted by endothelial cells such as endothelin-1 may inhibit adipose differentiation[17] and that conditioned medium from cultures of human adipose tissue endothelial cells promote preadipocyte proliferation.[18] In the current study, however, there was no relationship between the percentage of contaminating endothelial cells and the proliferation rate of the stromal cells nor differentiation capacity of the preadipocytes. Thus, further investigations are required to elucidate the influence of endothelial cells on preadipocyte differentiation and proliferation capacity. On the other hand, the observed lack of effect of endothelial cells on proliferation suggests that the difference found in proliferation capacity between subcutaneous and omental stromal cells is not due to a difference in the number of endothelial cells between these regions.

An additional observation was a negative correlation between age and proliferation of subcutaneous stromal cells but not of omental cells. A dependence of proliferation of stromal cells on age has been shown previously.[19,20] However, a novel finding may be that this relation might not be true for the omental region. This observation could be important as studies in humans suggest that aging is associated with a preferential growth of the omental adipose tissue in both men and women.[21-23] This underlines the role of regional differences in adipose tissue growth.

There were no major gender differences with regard to preadipocyte differentiation and proliferation in this study. The correlations found for the whole subject group were also found when the men were excluded from the analysis. These correlations were not found in the male group, probably because this group was too small to perform any valid linear regression analysis.

In conclusion, the results of this study indicate that there may exist regional differences in adipose tissue growth with special reference to proliferation capacity, whereas no substantial difference in differentiation capacity between subcutaneous and omental preadipocytes was observed. A clinically interesting aspect was that cell proliferation decreased with aging only in the subcutaneous but not the omental depot, which could contribute to the preferential expansion of the omental adipose tissue depots with age.

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